PCT / SE 2005 / 000252
23 -02- 2005

PA 1264891

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APPLICATION NUMBER: ***60/546,966***
FILING DATE: ***February 24, 2004***

PRIORITY DOCUMENT
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13281 U.S.PTO

MS PROVISIONAL PATENT APPLICATION
PTO/SB/16(8-00)

22154 U.S. PTO
60/546966
022404

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53 (c).

Filing Date	February 24, 2004	Docket No.	1291-0214P

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TITLE OF THE INVENTION (280 characters max)

NEW COMPLEXES

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages:* Seven (7)
☐ Drawing(s) *Number of Sheets:* _____
☐ Application Data Sheet. See 37 CFR 1.76.
☐ Other (specify):_____

METHOD OF PAYMENT *(check one)*	PROVISIONAL FILING FEE
☒ Applicant claims small entity status. See 37 CFR 1.27.	☒ Small Entity ($80.00)
☒ A check or money order is enclosed to cover the Provisional filing fees.	☐ Large Entity ($160.00)
☐ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 02-2448, if necessary.	

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

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1291-0214P

(Rev. 02/12/2004)

NEW COMPLEXES

ABSTRACT

The invention is related to new stable colloidal complexes between a charged peptide and a galactolipid. The new complex can be used as a drug delivery system for a charged peptide or as a stability ingredient for the preservation of the activity of a sensitive biologically active peptide.

FIELD OF THE INVENTION

The present invention relates to new complexes comprising charged bioactive compounds, such as peptides and proteins, and a neutral bilayer-forming lipid in aqueous solution. More specifically, the present invention refers to the use of new complexes as drug delivery systems for soluble drugs, particularly peptide drugs. The novel drug delivery system retards degradation of the drug, reduces toxicity, prevents adsorption of the drug to non-biological surfaces, and provides for sustained release of the incorporated drug. The system can be used to improve oral absorption of said bioactive compound and improve its transport through biological membranes. The delivery system can also be used for subcutaneous delivery of biological active peptides or in local delivery by means of bioadhesive adsorption. The said complex prevents degradation of said bioactive compound and stabilizes the drug.

BACKGROUND OF THE INVENTION

Antimicrobial peptides are highly charged effector molecules of the innate immune system, which serve to protect the host against potentially harmful microorganisms. They are conserved through evolution and are widespread in nature. In human, only a handful has been identified so far; among which the defensins and the human cathelicidin antimicrobial peptide hCAP18 have been implicated in epithelial defence (Selsted *et al.*, *J Biol Chem* 258:14485-14489, 1983). It has been proposed that cationic peptides first interact with micro-organisms by binding to their negatively charged surfaces, and for Gram-negative bacteria they act as outer membrane permeabilizers.

WO 96/08508 relates to the human polypeptide FALL-39, as well as to pharmaceutical compositions containing said peptide and having an antimicrobial activity against bacteria. The peptide was named FALL-39 after the first four amino acid residues and consisted of the 39 amino acid C-terminal part of a proprotein concomitantly identified by three separate

groups (Cowland *et al., FEBS*, 1995; Agerberth *et al., Proc Natl Acad Sci USA* 1995; Larrick *et al., FEBS Letters* 1996). The peptide was shown to have potent antimicrobial activity against both gram-positive and gram-negative bacteria. Further characterization of the C-terminal peptide demonstrated a shorter sequence comprising 37 amino acids excluding the first two (FA) resulting in LL-37, which is the accepted current designation (Gudmundsson *et al., Eur J Biochem* 238:325-332, 1996).

The proprotein was named hCAP18, Human Cationic Antimicrobial protein, and is a member of the cathelicidin family of proteins consisting of cathelin, which has been conserved through evolution and a C-terminal part, variable in different species. In man, hCAP18 is the only member of this protein family, whereas in other species, such as mouse and pig, there are several members. The C-terminal peptide LL-37 is thought to function extracellularly and there is no evidence for intracellular cleavage of the pro-protein. hCAP18/LL-37 is present in leukocytes and in barrier organs such as skin, mucous membranes, respiratory epithelium and reproductive organs. The localization of hCAP18/LL-37 to barrier epithelia seems to be consistent with a protective role for the peptide in preventing local infection and systemic microbial invasion. LL-37 is described as a cysteine-free peptide that can adopt an amphiphatic, or in other words amphiphilic, α-helical conformation. A high cationicity in combination with a stabilized amphiphatic α-helical structure seems to be required for the antimicrobial effect of such peptides against gram-positive bacteria and fungi, as has been shown experimentally (Gianga-spero *et al., Eur J Biochem* 268:5589-5600, 2001). The amphiphatic and α-helical structure seems to be less critical for killing of gram-negative bacteria. In association with inflammation hCAP18/LL-37 is up-regulated in skin epithelium (Frohm *et al., J Biol Chem* 272:15258-15263, 1997) and mucous membranes (Frohm Nilsson *et al., Infect Immun* 67:2561-2566, 1999).

Other charged peptides with antibacterial activity are gramicidin S, magainin, cecropin, hyphancin, cinnamycin, burforin I, parasin I and protamines. Cationic peptides, such as indolicidin with antifungal activity is also known as well as ApoA-I, ApoA-II, ApoA-IV, ApoC-I, ApoC-II, ApoC-III, ApoE, or other apolipoprotein analogues. Although there are a number of delivery systems which have been presented for peptides in general, none has been found to be useful for these cationic peptides or for other highly charged peptides.

In US 6,287,590 is shown a method to form a peptide lipid complex by co-lyophilization using one or more lipids in a solvent system. The lipids are selected from the group consisting of various charged phospholipids.

It has recently been demonstrated (PCT/SE04/00111) that mixtures consisting of an amphiphatic, positively charged peptide, LL-37, and a polar but neutral bilayer-forming lipid material, galactolipids, unexpectedly form stable, clear colloidal solutions at certain weight ratios. It was not possible to form corresponding solutions using pure phospholipids, a lipid material common in pharmaceutical applications, particularly in advanced drug delivery systems such as liposomes. Galactolipid is chemically digalactosyl diacylglycerol [1,2-diacyl-3-O-(α-D-galactopyranosyl-(1-6)-O-β-D-galactopyranosyl-glycerol), which can be obtained from Sigma Chemical Company, Miss. USA.

The appearance of the resulting solutions indicated that the peptide and the lipid formed complexes that were smaller in size than corresponding peptide-free liposomes. Colloidal solutions are per definition thermodynamically stable, and unlike liposomal dispersions, they do not separate on storing.

Furthermore, it was shown in PCT/SE04/00111 that the *in vitro* cytotoxicity of LL-37 was reduced when complexed with galactolipids.

DESCRIPTION OF THE INVENTION

The use of galactolipid-based liposomes in pharmaceutical applications has been described in WO 95/20944. This application does not disclose the use of galactolipids in combination with peptides and proteins in general, particularly not for forming complexes in solution, i.e. clear colloidal solutions, which are physically stable.

The present invention discloses stable galactolipid-peptide colloidal solutions, where the galactolipid and the peptide form a complex at certain weight ratios. The peptide shall have a molecular weight of less than 30 kDa to form a stable complex. Preferred peptides or proteins are those containing amino acid residues, which are positively charged. Lysine, arginine, histidine and ornithine are all naturally occurring amino acids, having basic side chains, which are positively charged at pH 7. Synthetic amino acids, which are positively charged at neutral pH are also possible to incorporate in a synthesized peptide, which are also disclosed in the

present invention. Furthermore, preferred peptides or proteins are those which have four or more positively charged amino acids. The charged amino acids should not be consecutive having sequences such as Lys-Arg-Lys-Arg.

Peptides with negative charged amino acids such as aspartic acid, glutamic acid or gamma-carboxy-glutamic acid are also disclosed in the present invention. The negatively charged amino acids should not be consecutive (Asp-Glu-Asp-Glu).

Preferably, the peptide or protein to be combined with the galactolipids is amphiphilic and surface active. Besides a charged portion the molecule also consists of a nonpolar portion. This may give rise to specific secondary structures in aqueous solution, as well as to aggregate formation (self-association) in aqueous solution.

Examples of peptides and proteins to be used in accordance with the present invention are, for example, those which form secondary structures in aqueous solution, structures such as α-helices, β-pleated sheets and the like. Specific examples are the cathelicidins including human cationic antimicrobial protein (hCAP18) and its C-terminal peptide LL-37, PR-39, prophenin, indolicidin, the latter which is a cationic tridecapeptide amide with a potent ant fungal activity; gramicidin S, a cyclic decapeptide with antibacterial activity; magainin; cecropin; hyphancin; cinnamycin; burforin I; parasin I; protamines; ApoA-I, ApoA-II, ApoA-IV, ApoC-I, ApoC-II, ApoC-III, ApoE, or other apolipoprotein analogues. Apo AI, is a single polypeptide with a molecular weight of 28 kDa. Its primary function is to activate LCAT (lecithin-cholesterol acyl transferase) within the HDL (high density lipoprotein) complex, which catalyzes the esterification of cholesterol.

Peptide hormones, such as motilin are also included in the group of peptides, which can be used according to the invention. Motilin is a 22 amino acid peptide secreted by endocrinocytes in the mucosa of the proximal small intestine. Motilin participates in controlling the pattern of smooth muscle contractions in the upper gastrointestinal tract.

Oligonucleotides, composed of charged nucleic acid resudues, are also possible to use according to the invention. Examples of therapeutic active agents based on oligonucleotides are the antisense agents, designed to specifically recognise and inhibit messenger RNA, thereby interacting with the synthesis of proteins in ribosomes.

Suitable counterions are acetate, chloride, etc, for a positively charged peptide, and sodium, potassium, ammonium, etc. for a negatively charged peptide.

The galactolipid from Sigma was obtained by purification from whole wheat flour. Galactolipids from any source including synthetic compounds can be used in the invention. The preferred tested lipid was a galactolipid material referred to as CPL-Galactolipid, manufactured by LTP Lipid Technologies Provider AB, Sweden. This is a purified galactolipid fraction from oats.

A suitable aqueous medium for the complexes is phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM NaCl, pH 7.4). However, any other aqueous solution with comparable ionic strength and appropriate pH may be used for the preparation.

The composition can in addition comprise pharmaceutically acceptable excipients, such as a preservative to prevent microbial growth in the composition, antioxidants, additional isotonicity agents, colouring agents, stabilising agents such as non-ionic surfactants and hydrophilic polymers, and the like.

DETAILED DESCRIPTION OF THE INVENTION

A stable peptide-galactolipid complex in aqueous solution is formed by the following general procedure:

The peptide and the galactolipid are weighed in a 100 ml glass flask and then PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.4) is added. The total volume is about 30 ml. The sample is vigorously shaken, using a suitable shaker at high speed, for 1-2 h or until the mixture has become clear, and is then allowed to equilibrate and settle for about 30 min at room temperature. Optionally, the clear solution is subjected to extrusion through a polycarbonate membrane with a pore size of 100 nm or less, in order to remove large complexes.

It should be noted that the procedure does not involve the use of ultrasonicators, high-speed mixers (ultra-turrax), high-pressure homogenisers, or other processing equipment, which is a clear advantage from a technical and economical point of view. Furthermore, it does not

require heat treatment, which makes it possible to prepare compositions containing heat sensitive bioactive compounds.

The colloidal nature of the composition makes it possible to prepare it aseptically by employing a final sterile filtration step. This is especially advantageous if the composition contains a bioactive molecule which is heat sensitive and thus not possible to heat sterilise.

The interactions between the charged peptide and the neutral lipid are sufficiently strong to accomplish a stabilization of the peptide and protect it from degradation both *in vitro* and *in vivo* through the complex formation. However, the interactions are weak enough to release the peptide from the complex once it has been delivered to the site of action. A charged (zwitterionic) phospholipid leads to too strong electrostatic interactions with the peptide. As a consequence the complexes tend to precipitate, more or less immediately after preparation. If at all possible to formulate and administer, there is then a potential risk of a too slow release of the peptide due to the strong forces between the charged phospholipid and the charged peptide.

The CPL-Galactolipid, which is based on an extraction of natural galactolipids from oats, is already used in dermatological creams and has been shown to be well tolerated, and to have good absorption properties. CPL-Galactolipid is stable at ambient temperature. Based on the experiments with the complex comprising LL-37 and the pharmaceutical properties of CPL-Galactolipid it can be concluded that the complex can be administered topically during long periods of time.

The present invention is not limited in scope by these described examples. Various modifications of the invention will be apparent to those skilled in the art. It is thus anticipated that it should be possible to form similar complexes based on galactolipids using other bioactive compounds having molecular weights less than 30 kDa, and being amphiphilic with a net charge. The optimal conditions, that is, weight ratio of compound to galactolipid and total concentration of the two ingredients are obtained by experiments. The aqueous solution should have an appropriate composition, ionic strength and pH as described above. The best composition for each unique peptide and galactolipid mixture is thus established and validated by means of the technically simple procedure described above.

CLAIMS

1. A galactolipid-peptide complex in solution having a galactolipid:peptide weight ratio of 1:5 – 1:50 wherein said peptide has a molecular weight less than 30 kDa; wherein said peptide is a charged amphiphilic polypeptide.
2. The complex according to claim 1 where the peptide has at least four positively charged amino acids.
3. The complex according to claim 1 where the peptide is in the form of a pharmaceutically acceptable salt.
4. The complex according to claim 1 where the galactolipid is CPL-Galactolipid.
5. The complex according to claim 1 where the peptide is in the form of acetate.
6. The complex according to any of the proceeding claims where the peptide is an antimicrobial cathelicidin peptide.
7. The complex according to any of the proceeding claims where the peptide is LL-37.
8. The complex according to any of the proceeding claims where the peptide is an apolipoprotein or an apolipoprotein analogue.
9. The complex according to any of the proceeding claims wherein the ratio between the peptide as a salt and the galactolipid is 1:10 – 1:50 by weight.
10. The complex according to any of the proceeding claims for use as a medicament.
11. The complex according to any of the proceeding claims for use as a medicament in wound healing.
12. A method of preparing a galactolipid-peptide complex which comprises adding the galactolipid to a peptide buffer solution, mixing the components with a mixer until the solution is clear and optionally filtering the solution through a sterile filter.
13. The method according to claim 12 wherein the peptide is a lipid binding polypeptide.
14. The method according to claim 12 wherein the peptide is LL-37.